

An Improved Media System for High Regeneration Rates from Barley Immature Embryo-Derived Callus Cultures of Commercial Cultivars

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ABSTRACT

Improved plant regeneration from tissues of elite barley cultivars (*Hordeum vulgare* L.) will facilitate their genetic transformation. Significant improvements in green plant regeneration from tissues of 'Morex' and 'Harrington' were achieved by modifying iron and boric acid concentrations, and by adding 6-benzyl aminopurine (BAP) to the culture medium. The plant regeneration in response to various concentrations of eight micronutrients indicated that MS concentrations were appropriate for KH_2PO_4 , NaMoO_4 , MnSO_4 , KI , CoCl_2 , and ZnSO_4 . Increased H_3BO_3 (0.75 mM) and decreased FeSO_4 (0.05 mM) improved green plant regeneration. The addition of 0.1 mg/L BAP to maintenance media resulted in an increase in regeneration. The effect of these changes, in combination with previously reported improvements, was assessed by comparing regeneration responses of eight cultivars and elite breeding lines on the improved media versus the original MS-based media. Green plant regeneration was improved for all tested lines and cultivars, from an average of two green plants to 29 green plants per Petri dish. Regeneration rates from all elite lines and cultivars on the improved media exceeded that of the highly regenerable 'Golden Promise' on the original media, indicating that poor plant regeneration should no longer limit the transformation of modern North American cultivars.

TISSUE CULTURE is required for a number of in vitro techniques in barley, including transformation and in vitro selection. These techniques have the potential to assist in breeding improved barley cultivars but have been limited to cultivars that are amenable to the formation of highly regenerable tissues. Research on barley cultivars and breeding lines of commercial importance in North America (NA) has revealed poor in vitro performance with respect to plant regeneration from embryogenic callus (Bregitzer, 1992; Baillie et al., 1993) initiated and maintained on common media formulations such as modified MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968) or CC (Potrykus et al., 1979). Cultivars such as Golden Promise and Igri, which are popular transformation targets because of their ability to regenerate plants following extended periods of in vitro growth and selection, are not adapted to the NA barley growing region, do not meet agronomic and quality requirements, and are susceptible to many diseases found in the USA. and Canada (USDA, 2001). Introgression of genes into NA germplasm from genetically divergent (but regenerable) germplasm is undesirable from a NA breeders' perspective. Directing efforts at genetic modification of elite germplasm relevant to NA breeding programs is therefore desirable.

Media components that have been modified to improve barley tissue culture regeneration include increased copper concentrations (Dahleen, 1995), altered nitrate:ammonium ratios (Nuutila et al., 2000), reduced (2,4-dichlorophenoxy)acetic acid (2,4-D) concentration and the addition of BAP to initiation and regeneration media (Bregitzer et al., 1998; Cho et al., 1998), altered media sterilization methods (Bregitzer et al., 1998), and the substitution of maltose for sucrose (Bregitzer, unpublished results). Few of these improvements have been applied successfully to barley transformation experiments to insert new genes into commercially important cultivars (Lemaux et al., 1999). Most past and current barley transformation efforts utilize Golden Promise on standard MS media.

The ongoing goal of our research has been to improve plant regeneration from embryogenic callus of cultivars of commercial importance in NA on the basis of the regeneration responses of callus derived from the elite cultivars Harrington and Morex. These two cultivars are important representatives of the NA two-rowed and six-rowed malting barley germplasm pools, respectively, and are the current quality standards by which other barley cultivars and breeding lines are judged. In this paper, we report additional modifications to our media regime on the basis of the responses of Harrington and Morex to alterations in micronutrient concentrations. In addition, we demonstrate the general utility of these and other previously reported modifications by comparing the regeneration responses of additional elite barley cultivars and breeding lines on both the "improved" and "original" media regimes, and also to the responses of the regenerability standard Golden Promise.

MATERIALS AND METHODS

Single plant derived lines (SPDLs) were used for all tests to remove varietal heterogeneity as a potential source of variability. Harrington and Morex were used for tests of micronutrient and BAP concentrations. Seven additional cultivars—elite breeding lines of diverse pedigree were included in comparisons of original vs. improved media: Foster, Drummond, Conlon, Colter, 90Ab321, Baronesse, and Crystal, plus the highly regenerable cultivar Golden Promise. Explant donor plant growth conditions were as described in Bregitzer et al. (1998). Donor plant seed for the combined tests were planted in the greenhouse at Fargo on 15 Apr. 1999.

Callus was initiated from immature zygotic embryos as described by Bregitzer et al. (1995). Four embryos (1–3 mm in length) were placed with the scutellum down on 30 mL medium (pH 5.8) in 15- by 100-mm Petri dishes (Aberdeen) or 50 mL medium (pH 5.8) in 20- by 100-mm Petri dishes (Fargo). At the first transfer to fresh medium, the larger Petri dishes were used at both locations. Initial experiments of individual micronutrients (KH_2PO_4 , NaMoO_4 , MnSO_4 , KI , CoCl_2 , ZnSO_4 , H_3BO_3 , and FeSO_4) used 12 Petri dishes per treatment for

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Table 1. Micronutrient concentrations tested and their effects on barley green plant regeneration from immature-embryo-derived callus of the cultivars Morex and Harrington. Concentrations highlighted by underscoring are those found in Murashige and Skoog medium (1962).

Micronutrient	Experiment	Replicates	Micronutrient concentrations tested							
			Avg. no. plants regenerated/Petri dish†							
			mM							
KH ₂ PO ₄	1	2	0	0.025	0.125	0.250	0.625	<u>1.250</u>	12.50	62.50
			0.29	0.30	0.43	0.44	1.52	1.70*	1.44	0
NaMoO ₄	1‡	2	0	0.0005	<u>0.001</u>	0.005	0.010	0.050	0.100	0.500
			0.84	1.04	0.94	1.02	0.89	0.88	0.53	0
KI	1‡	2	0	0.0025	<u>0.005</u>	0.025	0.050	0.250	0.50	2.50
			2.94	3.25	2.81	3.28	3.35	3.12	2.35	0.33
MnSO ₄	1‡	2	0	0.01	0.02	0.05	<u>0.10</u>	0.50	2.50	10.0
			0.99	1.29	1.53	1.75	1.56	2.06	1.57	0.53
ZnSO ₄	1	2	0	0.006	0.015	<u>0.030</u>	0.150	0.30	1.50	3.00
			0.16	2.43	1.87	1.76	2.15	1.33	0.16	0
	2‡	2	0.006	0.015	<u>0.030</u>	0.075	0.150			
			2.18	2.56	2.57	2.08	1.54			
CoCl ₂	1	2	0	0.0001	0.0005	<u>0.001</u>	0.005	0.01	0.05	0.10
			1.20	1.04	1.08	0.95	1.36	1.09	0.25	0.09
	2‡	2	0.0001	<u>0.001</u>	0.0025	0.005	0.0075	0.01		
			8.01	7.71	8.14	6.99	8.04	7.01		
FeSO ₄	1	2	0	0.01	0.02	0.05	<u>0.10</u>	0.50	2.50	10.0
			0.21	1.27	1.44	1.95	1.37	0.35	0	0
	2	4	0.02	0.035	0.05	0.075	<u>0.10</u>			
			4.50	5.55	5.89*	4.34	4.55			
H ₃ BO ₃	1	2	0	0.01	0.02	0.05	<u>0.10</u>	0.50	2.50	10.0
			0.31	0.71	0.51	0.64	0.82	1.08	0.43	0
	2	2	<u>0.10</u>	0.25	0.50	0.75	1.00			
			5.80	6.37	5.68	7.58	8.14			
	3	2	<u>0.10</u>	0.75	1.00	1.50	2.00			
			24.60	27.88*	23.36	24.76	9.93			

* Significantly different from other concentrations ($P = 0.05$) for a specific micronutrient experiment.

† Combined over locations and cultivars.

‡ No significant differences between concentrations ($P = 0.05$) when very high or low concentrations removed.

each cultivar. Subsequent experiments testing ZnSO₄, H₃BO₃, and FeSO₄ used 15 Petri dishes per treatment per cultivar. Comparisons of improved and original media included 20 Petri dishes per treatment per cultivar. At least one replicate was conducted at each location for each micronutrient. Additional experiments were conducted at each location for CoCl₂, ZnSO₄, FeSO₄, and H₃BO₃ to further define optimal concentrations. The tested micronutrient concentrations for each experiment are shown in Table 1.

Initial media regimes for micronutrient tests were conducted as described by Bregitzer et al. (1998). These regimes resulted in very low regeneration rates, making detection of true differences difficult. As studies progressed, regeneration rates were increased by incorporating identified improvements, including higher copper concentrations (5 μ M), altered sterilization methods, and a reduction in the number of calli per plate. Specifically, initial experiments testing concentrations of KH₂PO₄, NaMoO₄, CoCl₂, H₃BO₃, and FeSO₄ used MS CuSO₄ levels (0.1 μ M). All subsequent experiments used 5 μ M CuSO₄. The media components in the third H₃BO₃ experiment were autoclaved in three parts. The number of calli transferred to fresh media on each petri dish was reduced for all second and third experiments for a micronutrient. Final experiments combined the results from the micronutrient tests, the improvements described above, plus altered transfer times and plant growth regulator type and concentrations (Bregitzer et al., 1995, 1998; Cho et al., 1998; Dahleen, 1995) into an improved media system (Table 2) to compare with regeneration from the original MS-based media system that is used in most published barley in vitro experiments (similar to that reported by Bregitzer, 1992). Silver nitrate (AgNO₃) was included in regeneration medium at Aberdeen but not at Fargo. One experiment was conducted at each location. After 3 wk on initiation medium, half of the calli from three of the

embryos from each Petri dish were transferred to maintenance medium, again under dim lights. Calli were kept on maintenance medium for 6 wk, with one transfer to fresh medium after 3 wk. At the end of 6 wk, calli were moved to regeneration medium and placed under lights (3.1–5.5 μ mol m⁻² s⁻¹, warm and cool white fluorescent lamps), on a 16 h light/8 h dark cycle at 21 to 24°C. At Aberdeen, approximately one-third to one-half of the callus tissue was transferred to a single Petri dish; at Fargo, all of the callus tissue was transferred at regeneration to one to five Petri dishes, depending on the amount of tissue. Four weeks after transfer, albino and healthy green plants were counted and unrooted shoots and green sectors transferred to rooting medium. After another 4 wk, plants were again counted and added to the initial counts to give total regeneration per Petri dish.

An additional experiment was conducted to determine the effects of adding 0.1 mg/L BAP to the maintenance medium. Immature embryos of Morex and Harrington were cultured as described above. All media included 5 μ M CuSO₄. After 6 wk on initiation medium (transfer to fresh medium at 3 wk), calli from 20 Petri dishes were placed on maintenance medium containing 0.1 mg/L BAP, and 20 were placed on medium lacking BAP. After 6 wk (transfer at 3 wk), calli were placed on regeneration medium lacking plant growth regulators, and subsequently on rooting medium as described above.

Data were not normally distributed, especially in experiments with low regeneration rates where more than half of the cultures in some tests did not regenerate any plants, precluding the use of analysis of variance or means separation tests. Experiments were analyzed by the SAS (SAS Institute, 1999) procedure CATMOD. This procedure provides chi square-based analyses of variance that cannot be used to generate multiple comparisons or confidence intervals. Contrasts were used to compare specific micronutrient concentrations.

Table 2. Composition of media that were compared in final experiments.

Original media		Improved media	
MS basal salts and additions [†] , including:		MS basal salts and additions [†] , including:	
0.1 μM CuSO_4		5.0 μM CuSO_4	
0.1 mM H_3BO_3		0.75 mM H_3BO_3	
0.1 mM FeSO_4		0.05 mM FeSO_4	
3.5 g Phytagel		3.5 g Phytagel	
Growth regulators		Growth regulators	
initiation	3 mg L^{-1} 2,4-D	initiation	3 mg L^{-1} 2,4-D
maintenance	1.5 mg L^{-1} 2,4-D	maintenance	3 mg L^{-1} 2,4-D + 0.1 mg/L BAP
regeneration	none	regeneration	0.1 mg L^{-1} BAP \pm 17 mg/L AgNO_3
rooting	none	rooting	none
Carbon source		Carbon source	
initiation	30 g L^{-1} sucrose	initiation	30 g L^{-1} maltose
maintenance	30 g L^{-1} sucrose	maintenance	30 g L^{-1} maltose
regeneration	30 g L^{-1} sucrose	regeneration	30 g L^{-1} maltose
rooting	30 g L^{-1} sucrose	rooting	30 g L^{-1} sucrose
Coautoclave all components		Autoclave components in three parts (maltose; FeSO_4 , EDTA and KH_2PO_4 ; remaining components)	

[†] As modified by Bregitzer (1992).

Combined analyses were conducted on the identical experiments at each location treating each location as a replicate in the micronutrient and BAP experiments. Locations were analyzed separately in the final medium experiment.

RESULTS AND DISCUSSION

CATMOD analyses of all tested micronutrients showed significant differences ($P < 0.05$) attributable to concentration. Locations did not show significant differences ($P < 0.05$). Interactions between micronutrient concentrations and genotype were of no practical consequence, and the results presented below apply to both Morex and Harrington.

Tests of six of the eight micronutrients over a wide range of concentrations indicated that MS levels supported optimal plant regeneration (Table 1). Plant regeneration in response to various micronutrient concentrations either showed a clear peak at MS concentrations (KH_2PO_4), or showed no significant differences except at extremely high or low concentrations (NaMoO_4 , KI, CoCl_2 , ZnSO_4 , and MnSO_4). Removing data from very high or low concentrations resulted in the finding of no significant differences among the intermediate concentrations in subsequent CATMOD analyses.

Regeneration rates on KH_2PO_4 concentrations below 0.25 mM were approximately one quarter of the rates on MS concentrations (1.25 mM). This reduction in regeneration is similar to that found by He et al. (1989) in wheat (*Triticum aestivum* L.). They found that the percentage of 10-wk-old callus that regenerated shoots on different KH_2PO_4 concentrations was reduced 15-fold when 0.31 mM KH_2PO_4 was used in stead of MS concentrations (1.25 mM). Results from the first experiments testing ZnSO_4 and CoCl_2 concentrations were highly variable, with peaks both above and below the MS concentration. Additional tests of these micronutrients (Table 1) indicated that there were no significant differences in regeneration rates over a 25- to 100-fold change in micronutrient concentrations. Unexpectedly, there was no difference in regeneration from callus grown on medium lacking KI or CoCl_2 versus callus grown on medium containing MS concentrations of

these micronutrients. The lack of clear differences in regeneration responses as affected by large changes in concentration for some micronutrients is surprising and probably accounts for the wide applicability of the MS medium to cereal tissue culture, despite its specific creation for the culture of tobacco (*Nicotiana tabacum* L.) tissues (Murashige and Skoog, 1962).

Initial experiments with FeSO_4 showed that plant regeneration rates were higher on concentrations lower than those in MS medium (0.10 mM). Further tests indicated that regeneration was significantly greater at 0.05 mM FeSO_4 than at other concentrations (Table 1). Results with H_3BO_3 indicated that 0.75 mM H_3BO_3 was more appropriate for barley green plant regeneration than MS concentrations (0.10 mM).

The addition of 0.1 mg/L BAP to maintenance medium resulted in significantly ($P < 0.01$) higher regeneration rates (avg. 10.01 green plants per Petri dish) than when BAP was omitted (avg. 2.91 green plants per Petri dish). Cho et al. (1998) examined a barley culture system that maintains plant tissues in a partially differentiated green state. They found that the inclusion of BAP in maintenance medium increased the frequency of highly regenerative green structures and green plant regeneration compared with cultures on medium lacking BAP. The results of our study indicate that BAP also increases regeneration from callus that is embryogenic and relatively undifferentiated compared with the cultures of Cho et al. (1998). As seen with the micronutrient tests, both Morex and Harrington responded similarly to the addition of BAP to the medium.

These changes in H_3BO_3 and FeSO_4 concentrations and the addition of BAP in maintenance medium were incorporated, along with improvements based on previous research (as outlined in the Introduction), into an improved media regime and tested against a regime based on the media formulations used by Bregitzer (1992; Table 2). Plant regeneration from all cultivars and breeding lines at both test locations was significantly higher ($P = 0.05$) on the improved media regime (Fig. 1), except for Crystal at Aberdeen (Table 3). Combined over locations and genotypes, green plant regeneration was 29 green plants per Petri dish, an almost 15-fold

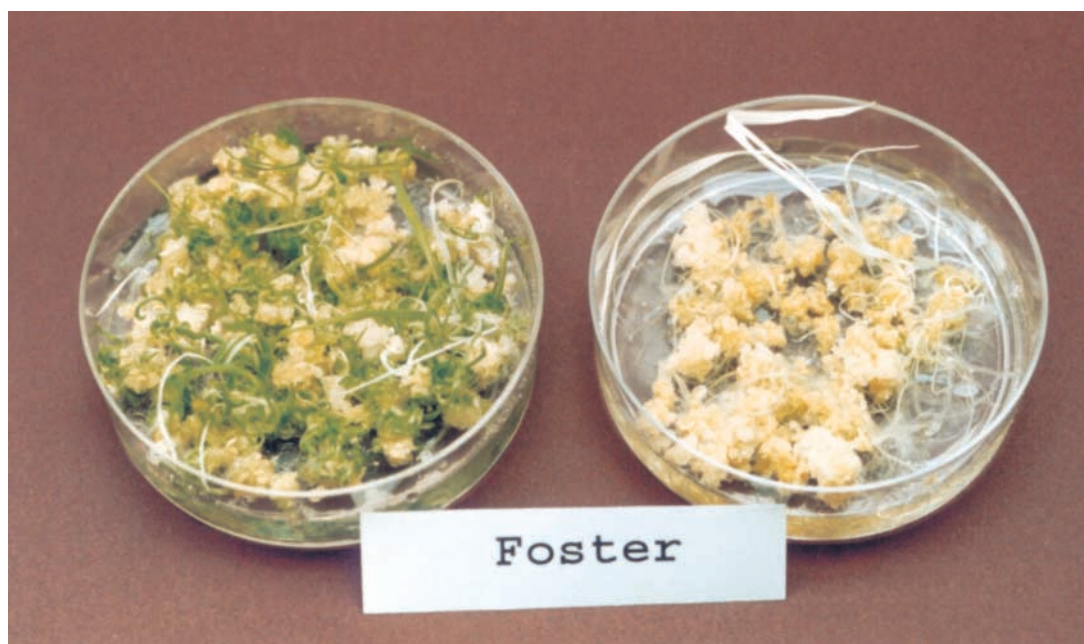


Fig. 1. Green and albino plant regeneration from immature-embryo-derived Foster barley calli grown on improved media (left Petri dish) and original media (right).

increase over the average of two green plants per dish on the original medium. This increase was much larger than the 4-fold increase in regeneration that resulted from only increasing copper concentrations in a test of 21 breeding lines and cultivars (Dahleen and Bregitzer, 1999). The increase in green plant regeneration in these cultivars on improved medium was not associated with an increase in albino plant regeneration (Table 3). Instead, the frequency of albino plants on improved medium was the same or lower than the frequency on original medium. This was especially true for Colter at Aberdeen, where the albino frequency was reduced from 9.8 per Petri dish on original medium to no albino plants per Petri dish on improved medium. The average frequency of albino plants was decreased, from 1.0 per Petri dish on the original medium to 0.4 on the improved medium.

On a given media regime, Golden Promise had the

highest green plant regeneration. However, the other breeding lines and cultivars all performed better on the improved medium than Golden Promise did on the original medium. In general, genotypes that had the highest regeneration rate on original medium showed a smaller increase in regeneration on improved medium than genotypes that had very low regeneration rate on original medium. For example, Baronesse, which had the second highest regeneration on original medium, showed only a 7-fold increase on improved medium while Conlon, which had low regeneration on original medium, had a 40-fold increase in regeneration on improved medium.

The numbers of recovered green plants were higher at the Fargo test location than at Aberdeen for all cultivars except Foster (Table 3). Regeneration rates at Fargo averaged 1.4-fold higher on original medium and 4-fold higher on improved medium as compared to regenera-

Table 3. Average number of green and albino plants regenerated per Petri dish from immature embryo-derived barley callus on original and improved media.

Genotype	Fargo				Aberdeen			
	Original media		Improved media (- AgNO ₃)		Original media		Improved media (+ AgNO ₃)	
	Green	Albino	Green	Albino	Green	Albino	Green	Albino
Morex SPDL2	0.3	0	38.9*	0.1	1	2.4	13.4*	1.1
Harrington SPDL1	0.8	0.3	27.9*	0	0.5	0.8	3.9*	0.6
Golden Promise SPDL8	10.5	0	101.7*	0	3.3	1.9	31.9*	1
Foster SPDL3	0.4	0.1	12.6*	0	0.4	0.8	13.3*	1.4
Drummond SPDL1	1.6	0.1	68.8*	0	1.7	0.5	7.8*	0.5
Conlon SPDL3	0.4	0.4	22.6*	0	0.6	0.3	16.5*	0.4
Colter SPDL1	1.1	0.2	61.1*	0	2.9	9.8	7.7*	0
90Ab321 SPDL1	1.3	0.1	25.3*	0.1	0.9	1.1	13.8*	1.1
Baronesse SPDL1	3.4	0	45.6*	0	5.0	1.2	10.4*	0.4
Crystal SPDL1	2.6	0	53.5*	0.3	0.8	0.7	2	0.6
Averages	2.3	0.1	45.8	0	1.7	1.9	12.1	0.7

* Significantly more green plants ($P = 0.05$) regenerated on improved media than on original media.

tion rates at Aberdeen. The most probable source of this difference was that at Fargo all callus tissue present at the end of the maintenance period was transferred to regeneration medium, and this tissue was apportioned between multiple (as many as five) Petri plates. At Aberdeen, approximately one-third to one-half of the callus tissue was transferred from maintenance to regeneration medium. Another component in the differences between Aberdeen and Fargo results may trace to genotype by environment interactions of the donor plants, which ultimately affect callus growth. It has been shown that immature embryos obtained from donor plants grown under optimal greenhouse growth conditions (versus growth chamber conditions) can produce callus cultures with higher rates of regeneration (Dahleen, 1999). Finally, silver nitrate was used in the regeneration medium at Aberdeen but not at Fargo. Silver nitrate has been reported to have a stimulatory effect on plant regeneration (Purnhauser et al., 1987) for several species, but discovering an appropriate method for stimulating plant regeneration from barley callus has remained elusive (Bregitzer, unpublished results). In the present study, we could not detect either a consistent inhibitory or stimulatory effect of silver nitrate. Genotypic variability in endogenous ethylene production has been observed and related to differences in somatic embryo formation (Cho and Kasha, 1989), and appropriate use of ethylene inhibitors such as silver nitrate may have to be specifically tailored for a particular cultivar.

A period of 6 wk on maintenance medium was used for these experiments, which is a shorter period than typically used to select transgenic barley calli. Additional experiments (data not shown) indicate that high levels of regenerability are retained by calli maintained for 12 wk or longer. A small number of regenerants from these experiments were grown to maturity in the greenhouse. Plants had normal fertility and seed set, and generally did not show any gross phenotypic abnormalities.

The justification for the use of Golden Promise for routine transformation projects has been its very high rates of regeneration. Problems with regeneration of transformed barley cells have long been a weak link in the chain of events that must occur to successfully produce transgenic barley plants. We now have a media regime that will produce plant regeneration rates, from a wide range of germplasm important to North America, that exceed those that have been a part of successful transformation protocols using Golden Promise. Although the regeneration rate of Golden Promise remains the highest on the improved medium, which may justify its continued use for certain experiments, the use of the new medium will allow the production of transgenics directly in commercially important germplasm.

ACKNOWLEDGMENTS

This work was partially supported by the American Malting Barley Association, Inc., Milwaukee, WI. The authors thank Mrs. Luming Brewer and Mr. Robert Campbell for their expert manipulations of the tissue cultures during these experiments, and Gary Richardson and Mark West for their assistance on statistical analyses.

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